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# Transcriptional activation of the *Prox1* gene by HIF-1α and HIF-2α in response to hypoxia

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ABSTRACT

***Prox1* encodes a homeobox transcription factor critical to organ development, but its regulation is poorly understood. Here, we show that *Prox1* expression is induced by hypoxia, and controlled by a hypoxia-response element (HRE) at the *Prox1* promoter/regulatory region and HIF-1α/HIF-2α. EMSA and ChIP assays demonstrated the direct interaction of the HRE with HIF-1α or HIF-2α. Overexpression of HIF-1α or HIF-2α increased activation of the *Prox1* promoter, whereas knockdown of HIF-1α or HIF-2α inhibited the activation. These data reveal a novel molecular mechanism for regulation of *Prox1* expression in response to hypoxia and provide new insights into *Prox1*-controlled processes such as lymphangiogenesis.**


## 1. Introduction

The *Prox1* gene encodes a homeobox transcription factor that plays an important role in organ development during embryogenesis [1]. The human *Prox1* gene is located on chromosome 1q32.2–q32.3 and composed of 5 exons and 4 introns [2]. The Prox1 transcription factor contains 737 amino acid residues and has an estimated molecular weight of 82.3 kDa [2]. Structurally, Prox1 contains a nuclear localization signal (NLS) and two nuclear receptor boxes (NR Box) at the N-terminus, and an atypical homeodomain followed by a prospero domain at the C-terminus [3]. The homeobox and prospero domain are responsible for DNA binding and the NR boxes can interact with nuclear receptors such as HNF4a/NR1A1, LRH-1/NR5A2 (FTF) and SF-1/NR5A1 [4]. The prospero domain contains a PCNA interacting protein motif [5]. The transcriptional activation domain of Prox1 has not been identified yet.

*Prox1* is critical to embryonic morphogenesis and development [1] as *Prox1* knockout mice die at E15 before birth [6]. *Prox1* is best known as a marker for monitoring the development of the lymphatic vasculature in various tissues [7,8]. *Prox1* knockout embryos lacked the lymphatic vasculature although vasculogenesis and angiogenesis were not affected in these embryos, suggesting that it is required specifically for the development of the lymphatic system [3,6,7]. Dudas et al. showed that *Prox1* was a hepatocyte marker during liver development, and the liver was 70% smaller in *Prox1* knockout embryos than in their wild type counterparts due to lack of hepatocytes [4,9–12]. *Prox1* was also involved in the development of the heart. Mice with cardiac-specific knockout of *Prox1* had 30% reduction in the size of the heart [13]. In addition, *Prox1* has been shown to be critical to the development of the pancreas, lens, spinal cord, and the central nervous system [11,14–16].

Despite the importance of *Prox1* as a key regulatory protein critical to the development of various organs, little is known about how *Prox1* expression is regulated. Hypoxia is an important physiological stimulus often associated with a variety of pathological states [17]. HIF-1α and HIF-2α are the pivotal mediators of the cellular responses to hypoxia, and play major roles in development, physiology and pathophysiology [18–20]. The HIF transcription factors are basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) proteins that form heterodimeric complexes composed of an $O_2$-labile α-subunit (e.g. HIF-1α, HIF-2α) and a stable β-subunit (HIF-1β) [18]. These subunits bind hypoxia-responsive elements (HREs) that contain a conserved RCGTG core sequence. HIF-1α and HIF-2α subunits are structurally similar in their DNA binding and dimerization domains, but differ in their transactivation domains [21]. Here, we show that hypoxia can induce the expression of *Prox1*

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and this response was mediated by HIF-1α and HIF-2α at the transcriptional level.

## 2. Materials and methods

### 2.1. Plasmids and siRNAs

Using human genomic DNA, we amplified the proximal promoter region of *Prox1* (−1500 to +300 flanking the transcription start site, TSS) that contains a consensus DNA binding site for HIF-1α/HIF-2α (HRE) by PCR analysis. The PCR primers included a forward primer of 5′-GCGCGCGGTACCCCAGATGTTTGCAACATA-TA-3′ and a reverse primer of 5′-GCGCGCCTCGAGGCAGGAGAAA-GAAGGAAAGG-3′. The PCR product was digested with restriction enzymes *Kpn*I and *Xho*I and sub-cloned into the multiple cloning site of the pGL3-basic luciferase vector cut with the same enzymes. In this construct, the *Prox1* promoter fragment was inserted upstream of the firefly luciferase coding region. Then, the HRE sequence was mutated from ACGTG into ACATA using PCR-based site-directed mutagenesis as described previously [22]. The primers used for mutagenesis included a forward primer 5′-TGTGACATACAGTCTTCCTGTT-3′, a reverse primer of 5′-CTGTATGTCACATCCTTCAGCT-3′, and the two PCR primers described above. The two resulting plasmids were referred to as WT-Prox1p-Luc and MUT-Prox1p-Luc, respectively.

Information on other plasmids and siRNAs can be found in online Supplementary information.

### 2.2. Luciferase assays

Luciferase assays were carried out as described previously by us [22,23]. Each experiment was performed in triplicate and repeated at least three times. Details are in online Supplementary information.

### 2.3. Hypoxia treatment

Hypoxic conditions were induced by culturing the cells in a standard hypoxic exposure condition with 3% $O_2$, 5% $CO_2$, and 92% $N_2$ in a hypoxic incubator (Heal Force, HF100, Shanghai, China) for 48 h. Hypoxic conditions were also mimicked by adding to culture media 100 μM of hypoxia-mimetic agents desferrioxamine (DFX, Sigma–Aldrich, Hamburg, Germany), cobalt (II) chloride ($CoCl_2$, Sigma–Aldrich, Hamburg, Germany) or dimethyloxalylglycine (DMOG, Sigma–Aldrich, Hamburg, Germany) [24,25]. The cells were then incubated for 48 h under a normoxic condition (5% $CO_2$, 21% $O_2$, and balance $N_2$) in a humidified incubator (Forma scientific, Marietta, OH, USA).

### 2.4. Real-time PCR analysis

Real time quantitative PCR analysis was carried out as described previously [23]. Details are in online Supplementary information.

### 2.5. Western blot analysis

Western blot analysis was carried out as described by us previously [26]. Details are in online Supplementary information.

### 2.6. Electrophoretic mobility shift assays (EMSA) for detecting protein–DNA interaction

EMSA were carried out using a LightShift chemiluminescent EMSA kit (catalog #20148; Thermo Scientific, Rockford, IL, USA) as described [27]. Details are in online Supplementary information.

### 2.7. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using the EZ-ChIP kit (Millipore, Billerica, MA, USA) as described [28]. ChIP assays were replicated three times. Details are in online Supplementary information.

### 2.8. Statistical analysis

The data were from three independent experiments, and presented as means ± standard deviation (S.D.). Statistical analysis was carried out with a Student's *t*-test using SPSS version 16.0 software (SPSS, Chicago, IL, USA). Differences were considered statistically significant when $P < 0.05$.

## 3. Results

### 3.1. Identification of a highly conserved hypoxia-response element (HRE) at the Prox1 promoter and regulatory region

To characterize the critical role of *Prox1* in lymphangiogenesis and development of other organs, we analyzed potential molecular mechanisms that regulate the expression of *Prox1* by focusing on its promoter and regulatory region. From DBTSS (Database of Transcriptional Start Sites, http://dbtss.hgc.jp/index.html?nmid=DBTSS:NM_002763), we identified the transcriptional start site (TSS) of *Prox1* at the physical position of 214,161,859 on human chromosome 1. The sequence for the *Prox1* promoter and regulatory region was retrieved from the TRED database (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=promInfo&pid=1871), and used for identifying DNA sequence motifs that bind to transcription factors using PROMO 3.0 and Contra V2. A Position Weight Matrix (PWM) for HIF-1α/HIF-2α was selected from the TRANSFAC collection and used for the analysis (Fig. 1A). The analysis led to the identification of an HRE for HIF-1α/HIF-2α located at −640 bp to −592 bp upstream of the *Prox1* TSS (Fig. 1B). Further analysis showed that the HRE was highly conserved among different species during evolution (Fig. 1B).

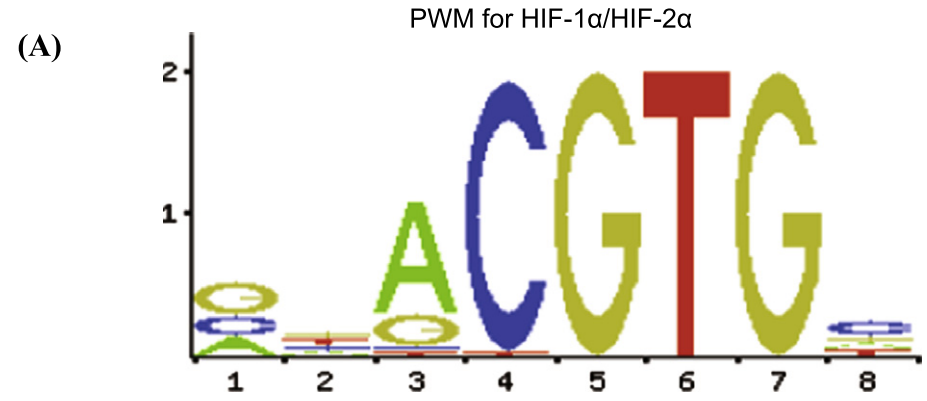
### 3.2. Hypoxia induces transcriptional activation of the Prox1 promoter

To test whether the HRE in the *Prox1* promoter/regulatory region is functional, we constructed a WT-Prox1p-Luc reporter gene in which a *Prox1* promoter/regulatory fragment (−1500 to +300 bp from TSS) was cloned upstream of the *luciferase* gene (*Luc*) (Fig. 2A). HeLa cells were transfected with the reporter, cultured under a normoxic condition or a hypoxic condition, and assayed for transcriptional activation of the *Prox1* promoter as represented by luciferase activity. As shown in Fig. 2B, hypoxia-mimetic agents $CoCl_2$, DFX and DMOG all increased the *Prox1* promoter activity by 2.5-fold compared to control PBS. Incubation of the transfected cells at a hypoxia chamber (3% $O_2$) increased the *Prox1* promoter activity by about threefold compared to under a normal culture condition (normoxia, 21% $O_2$) (Fig. 2C).

To determine whether the HRE is responsible for hypoxia-induced transactivation of the *Prox1* promoter, we mutagenized the HRE from ACGTG to ACATA in WT-Prox1p-Luc. The mutation abolished the response of the *Prox1* promoter to hypoxia (Fig. 2B and C). These results suggest that the HRE at the *Prox1* promoter/regulatory region is directly involved in regulating the transcriptional initiation of the *Prox1* gene.

### 3.3. Hypoxia induces up-regulation of Prox1 expression in vivo

To further validate the finding that hypoxia induces *Prox1* expression, we investigated whether the expression levels of




(B) Human_PROX1_NM_002763_Promoter_chr1:214161219-214161268

HIF binding site

| | |
|---|---|
| HUMAN_PROX1 | GCTGGGGC-------CGG-GTACG-TCAGATAGACTGTGACGTGC-AGTCTTCCTGTTT |
| BABOON | GCTGGGGC-------CGG-GTACG-TCAGATAGACTGTGACGTGC-AGTCTTCCTGTTT |
| CHIMP | GCTGGGGC-------CGG-GTACG-TCAGATAGACTGTGACGTGC-AGTCTTCCTGTTT |
| ORANGUTAN | GCTGGGGC-------CGG-GTACG-TCAGATAGACTGTGACGTGC-AGTCTTCCTGTTT |
| MARMOSET | GCTGGGGC-------CGG-GTACG-TCAGATAGACTGTGACGTGC-AGTCTTCCTGTTT |
| TARSIER | GTTGGGGC-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| MOUSE LEMUR | GCCGGGGC-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| BUSHBABY | GCTGGGGC-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| MOUSE | GCTGGGAC-------CGG-GTACG-TCAGTGGGACTGTGACGTGC-AGTCTTCCTGTTT |
| RAT | GCTGGGGC-------CGG-GTACG-TCAGTGGGACTGTGACGTGC-AGTCTTCCTGTTT |
| RABBIT | GCAGGGGA-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTCT |
| COW | GCTGGGGC-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| SHREW | GCTGGGCG-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-GGCCTTTCTGTTT |
| ROCK HYRAX | GCT-GGGC-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| ELEPHANT | GCT-GGGC-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| ARMADILLO | GCT-GCGT-------CGG-GTACGTTCAGTCAGACTGTGACGTGC-AGTCTTTCTGGTT |
| OPOSSUM | GCTGAGTC-------TGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| PLATYPUS | GCTGGGTC-------CGG-GTACG-TCAGTCAGACTGTGACGTGC-AGTCTTCCTGTTT |
| CHICKEN | GGCGGGCC-------GGGCTCACG-TCACCCGGCCCGTGACGTGT-AGTCTTCCTGTTT |
| LIZARD | GCCGGGCC-------TGGCGCACG-TCACTCAGGCCGTGACGTGT-AGTCTTCCTGTTT |

relative position to TSS (214161859) for reference species: -640 to -592

**Fig. 1.** Identification of a highly conserved HIF-1α/HIF-2α binding site (HRE) in the *Prox1* promoter region in different species by bioinformatic analysis. (A) A Position Weight Matrix (PWM) used for identifying HREs from the TRANSFAC collection. (B) A consensus HRE for HIF-1α/HIF-2α-binding was identified in the region −640 bp to −592 bp upstream of the transcription start site (TSS) at the proximal promoter of human *Prox1* gene. The sequence for the chromosome 1 region from 214,161,219 to 214,161,268 of the human *Prox1* promoter is shown and the HIF binding site is highlighted. The HREs at the *Prox1* promoters show high conservation among different species. The TSS is at the position of 214,161,859.

endogenous *Prox1* mRNA and protein were induced under hypoxic conditions. We selected four cell lines for this study, including human umbilical endothelial cells (HUVEC) in which *Prox1* was highly expressed, SY-SY5Y representing the neuronal system, HLE representing the lens system, and HepG2 representing the liver system. Endogenous *Prox1* expression was easily detected in all four cell lines (data not shown). The cells were incubated at either a normoxic condition or a hypoxic condition for 48 h, and then used for measuring the expression levels of *Prox1* mRNA or protein. Real-time PCR analysis showed that *Prox1* mRNA expression was dramatically increased by hypoxia (5- to 13-folds) in all four cell lines tested (Fig. 3A–D). By comparison, the magnitude of induction of positive control *VEGF-A* mRNA expression was considerably less than that for *Prox1* mRNA expression (Fig. 3A–D). On the protein level, Western blot analysis showed that the expression of Prox1 was markedly increased in response to hypoxia as compared to normoxia in all four cell lines (Fig. 3E–H).

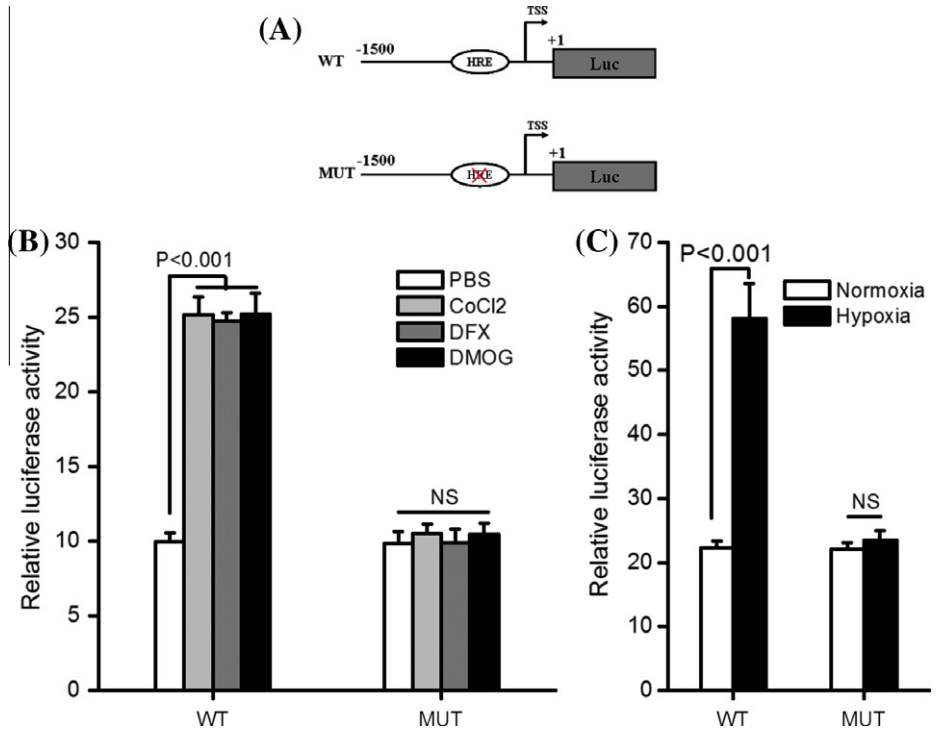



**Fig. 2.** Hypoxia significantly induces transcriptional activation of the human *Prox1* promoter. (A) Schematic diagram of the *Prox1* promoter luciferase reporter genes (*Prox1p-Luc)*. WT, wild type *Prox1p-Luc* reporter; MUT, mutant *Prox1p-Luc* with the HRE sequence mutated from ACGTG to ACATA. (B) Transcriptional activation activity (i.e. relative luciferase activity) for WT or MUT *Prox1p-Luc* reporters in HeLa cells treated with PBS buffer (negative control) or hypoxic mimetic reagents DFX, CoCl2 or DMOG for 48 h. (C) Transcriptional activation activity (i.e. relative luciferase activity) of WT or MUT *Prox1p-Luc* reporters in HeLa cells cultured under a normal condition (Normoxia) or in a hypoxic incubator (3% $O_2$, Hypoxia) for 48 h.

### *3.4. Hypoxia-induced activation of the Prox1 promoter is mediated by both HIF-1α and HIF-2α*

HIF-1α and HIF-2α are the two transcriptional factors that mediate the cellular responses to hypoxia. Therefore, we hypothesized that HIF-1α, HIF-2α, or both are involved in the transactivation of the *Prox1* promoter under hypoxia. To test the hypothesis, we co-transfected WT-Prox1p-Luc and a *HIF-1α* mammalian expression plasmid or a *HIF-2α* expression plasmid into HeLa cells and performed dual luciferase assays. As shown in Fig. 4A, overexpression of either HIF-1α or HIF-2α significantly increased the *Prox1* promoter activity compared to the empty vector control.

We also investigated whether the expression of endogenous *Prox1* mRNA in HLE cells was affected by overexpression of *HIF-1α* or *HIF-2α* by real-time qPCR and Western blot analyses. The expression level of endogenous *Prox1* mRNA increased significantly with overexpression of HIF-1α or HIF-2α (Fig. 4B). The *Prox1* mRNA expression appeared to be more responsive to *HIF-2α* than to *HIF-1α* (Fig. 4B). Western blot analysis showed that successful overexpression of either HIF-1α or HIF-2α also increased the expression level of Prox1 protein compared to the vector control (Fig. 4C).

To further validate the above finding that both HIF-1α and HIF-2α mediate the induced transcriptional activation of *Prox1*, we knocked down expression of *HIF-1α* and *HIF-2α* by specific siRNAs in HLE cells and incubated the cells under in a hypoxia chamber (a condition with increased HIF-1α or HIF-2α) for 48 h. The expression level of Prox1 was quantified by Western blot analysis. As shown in Fig. 4D, HIF-1α or HIF-2α expression was successfully knocked down by their respective siRNA compared to a universal scramble siRNA (Fig. 4D). Prox1 protein expression under hypoxia was down-regulated by either *HIF-1α* or *HIF-2α* siRNA. Knockdown of both *HIF-1α* and *HIF-2α* reduced Prox1 protein expression much more than knockdown of either one (Fig. 4D).

### *3.5. HIF-1α or HIF-2α interacts directly with the HRE at the Prox1 promoter*

To further explore the molecular mechanism by which hypoxia induces expression of *Prox1*, we performed EMSA using a biotin-labeled, double-stranded oligonucleotide probe containing the *Prox1* HRE located at the region of −640 bp to −592 bp upstream from the TSS (Fig. 5A). Incubation of the EMSA probe with proteins extracts isolated from HLE cells transfected with either HIF-1α or HIF-2α expression plasmids resulted in a protein–DNA complex formation (Fig. 5B, lane 2 and lane 4). The protein–DNA complex formation disappeared with addition of 200-fold excess of an unlabeled competitor, a double-stranded oligonucleotide probe containing the consensus HRE sequence (Fig. 5A and B, lane 3 and lane 5). The protein–DNA complex was specific to either HIF-1α or HIF-2α because it disappeared when protein extracts were pre-incubated with an anti-HIF-1α or anti-HIF-2α antibody in EMSA and a super-shifted protein–DNA complex was observed (Fig. 5B, lane 6 and lane 7). These data suggest that the *Prox1* HRE can interact directly with either HIF-1α or HIF-2α in vitro.

The in vitro EMSA results were validated by ChIP analysis using HLE cells treated with hypoxia, which demonstrated that endogenous, native HIF-1α or HIF-2α could bind to the *Prox1* promoter in vivo (Fig. 6). The protein–DNA complex between the *Prox1* HRE and HIF-1α or HIF-2α in HLE cells was immunoprecipitated using an antibody specifically against HIF-1α or HIF-2α or anti-rabbit IgG as negative control. The identity of the DNA in immunoprecipitates was identified by real-time PCR analysis using primers

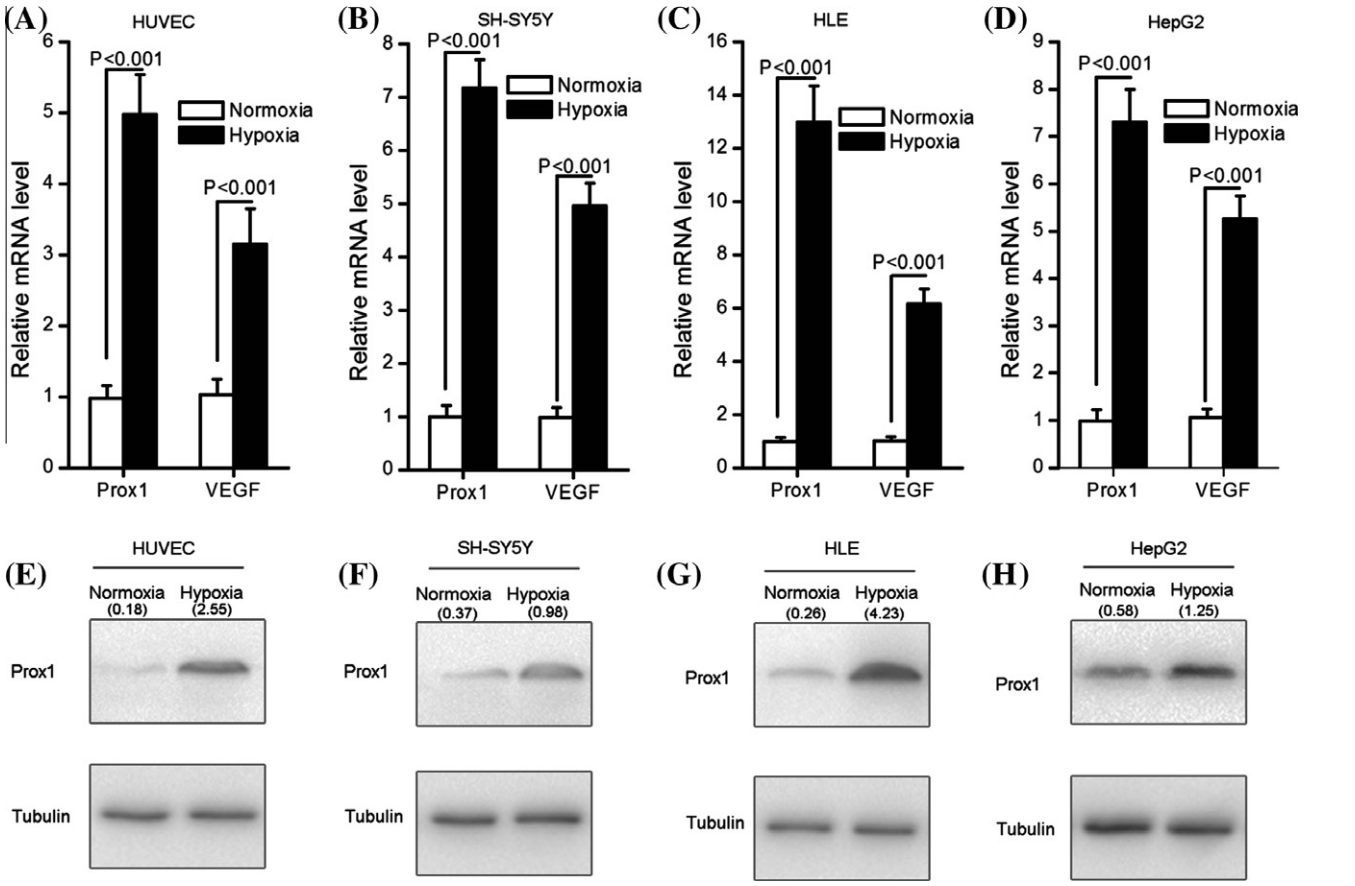



**Fig. 3.** Hypoxia induces expression of endogenous *Prox1* in four different cell lines, HUVEC, SY-SY5Y, HLE and HepG2. (A–D) Hypoxia dramatically stimulated expression of *Prox1* mRNA. HUVEC, SY-SY5Y, HLE and HepG2 cells were cultured under either normoxic or hypoxic conditions (3% $O_2$) for 48 h and then used for measuring the expression level of *Prox1* mRNA by real-time PCR analysis. The *VEGF-A* gene was used as a positive control. (E–H) Hypoxia stimulated expression of *Prox1* protein. Cells were treated as in A–D for 48 h, lysed and used for Western blot analysis to measure the expression level of Prox1 protein. Tubulin was used as the loading control. The relative intensity of the Prox1 band over that of the tubulin band was noted in brackets above the Western blot image.

specific for the *Prox1* HRE site. We detected significant enrichment of the *Prox1* HRE with either an anti-HIF-1α antibody or an anti-HIF-2α antibody, but not with control rabbit IgG (Fig. 6B). By contrast, real-time PCR analysis did not detect any PCR signal in a distal *Prox1* region without HRE (Fig. 6B). These data indicate that HIF-1α or HIF-2α can interact with the *Prox1* HRE in vivo.

## 4. Discussion

In this study, we characterized the regulatory mechanisms for the expression of the homeobox gene *Prox1*. We found that *Prox1* expression was dramatically increased under hypoxia. The hypoxia-response of *Prox1* was mediated by HIF-1α and HIF-2α, which are two pivotal mediators for cellular responses to hypoxia and play major roles in development, physiology and pathophysiology. Bioinformatic analysis identified one potential HRE for HIF-1α and HIF-2α at the promoter region of *Prox1* (Fig. 1). Luciferase assays using a *Prox1* promoter reporter showed that hypoxia induced transcriptional activation of the *Prox1* promoter. A HRE mutation from ACGTG to ACATA abolished the transcriptional activation of *Prox1* by hypoxia, suggesting that the HRE is essential for the activation (Fig. 2). Both EMSA and ChIP assays showed that HIF-1α and HIF-2α interacted with the HRE within the *Prox1* proximal promoter region (Figs. 5 and 6). Hypoxia dramatically increased *Prox1* expression at both the mRNA and protein levels (Fig. 3). In addition, *Prox1* expression was induced by overexpression of either HIF-1α or HIF-2α. Depletion of HIF-1α and HIF-2α abolished the induction of *Prox1* transcription under hypoxia (Fig. 4). These studies indicate that *Prox1* expression is regulated by hypoxia, HIF-1α and HIF-2α.

The regulation of *Prox1* is an area less well-characterized. In 2011, two independent studies started to shed some insights into the potential upstream regulators of *Prox1* in the neuronal system. In one study, the expression level of *Prox1* was increased in neuronal progenitor cells by twofold in *SOX1* knockout mice [29]. The study suggests that *SOX1* may regulate the expression of *Prox1*, but the molecular mechanism is not known, nor is whether the regulation is direct or indirect. In the second study, *Prox1* regulation was found to be positively regulated by two TCF/LEF sites in the *Prox1* regulatory region upon activation of the Wnt signaling pathway in the adult hippocampus [30]. The finding of direct transcriptional regulation of *Prox1* by HIF-1α and HIF-2α in the present study significantly expands the spectrum of upstream regulators of *Prox1* and provides novel insights into the regulatory mechanisms for *Prox1* transcriptional activation.

HIF-1α and HIF-2α are structurally and functionally similar, but not identical. They can modulate the transcriptional activation of a set of common genes, but each can regulate a distinct set of target genes [31]. Previously studies showed that the N-terminal activation domain (NAD) contributes to the target gene specificity by HIF-1α and HIF-2α, whereas the C-terminal activation domain (CAD) activates common target genes [18,31]. In the present study, we found that both HIF-1α and HIF-2α can individually activate *Prox1* expression (Fig. 4A–C). HIF-2α appeared to be a consistently

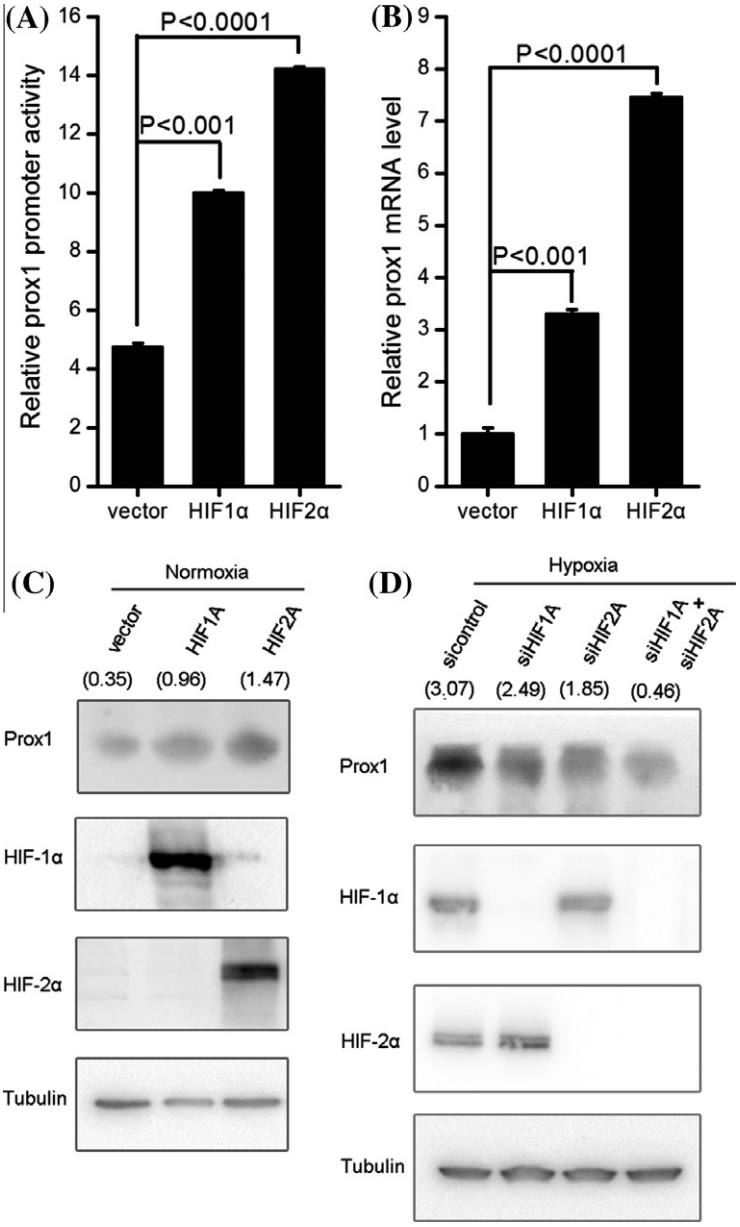



**Fig. 4.** Transcriptional activation of *Prox1* is regulated by both HIF1α and HIF2α. (A) Overexpression of HIF-1α or HIF-2α by transient transfection in HeLa cells for 48 h increased transcriptional activation activity (i.e. relative luciferase activity) of WT *Prox1p-Luc* reporter. Transfection with an empty vector was used as control. (B) Overexpression of HIF-1α or HIF-2α by transient transfection in HLE cells for 48 h increased the expression level of *Prox1* mRNA as measured by real-time PCR analysis. Transfection with an empty vector was used as control. (C) Overexpression of HIF-1α or HIF-2α by transient transfection in HLE cells for 48 h increased the expression level of Prox1 protein as measured by Western blot analysis. The expression level of HIF-1α or HIF-2α was also measured by Western blot analysis. Tubulin was used as the loading control. The relative intensity of the Prox1 band over that of the tubulin band was noted in brackets above the Western blot image. The experiments were performed under the normoxic conditions. (D) Knockdown of expression of HIF-1α and/or HIF-2α decreased the expression level of Prox1 protein. HLE cells cultured under hypoxic conditions (3% $O_2$) were transfected with HIF-1α siRNAs (siHIF1A), HIF-2α siRNAs (siHIF2A) or both siRNAs for 48 h, lysed and used for Western blot analysis to measure the expression levels of Prox1, HIF-1α and HIF-2α. sicontrol, universal scramble control siRNA. Tubulin was used as the loading control. The relative intensity of the Prox1 band over that of the tubulin band was noted in brackets above the Western blot image.

stronger activator for *Prox1* than HIF-1α, although the differences were significant in the real-time PCR analysis only ($P < 0.001$, Fig. 4B), but not in the luciferase assays or Western blot analysis ($p = 0.51$ in Fig. 4A, $p = 0.27$ in Fig. 4C). In the RNA interference studies, a marked decrease of *Prox1* expression was observed only when both HIF-1α and HIF-2α were knocked down (Fig. 4D), consistent with the finding that either HIF-1α or HIF-2α can activate *Prox1* expression (redundancy). Thus, HIF-1α and HIF-2α may mediate transcriptional activation of *Prox1* using the CAD. Moreover, expression of HIF-1α was more ubiquitous than HIF-2α. HIF-2α expression showed tissue-specificity, and is expressed only in certain tissues such as the endothelium, kidney, lung, heart, and small intestine [32]. Therefore, expression of *Prox1* may show tissue-specific patterns under hypoxia, and future studies are needed to test this hypothesis.

*Prox1* plays an essential role in lymphangiogenesis [33]. Although it is well-known that hypoxia and its effectors HIF-1α and HIF-2α can induce angiogenesis [18], its role in lymphangiogenesis is largely uncertain. Our results showing strong induction of *Prox1* mRNA and protein by hypoxia provide additional evidence that hypoxia may indeed promote lymphangiogenesis by activating *Prox1*. Recently, hypoxia was shown to influence lymphangiogenesis in the presence of

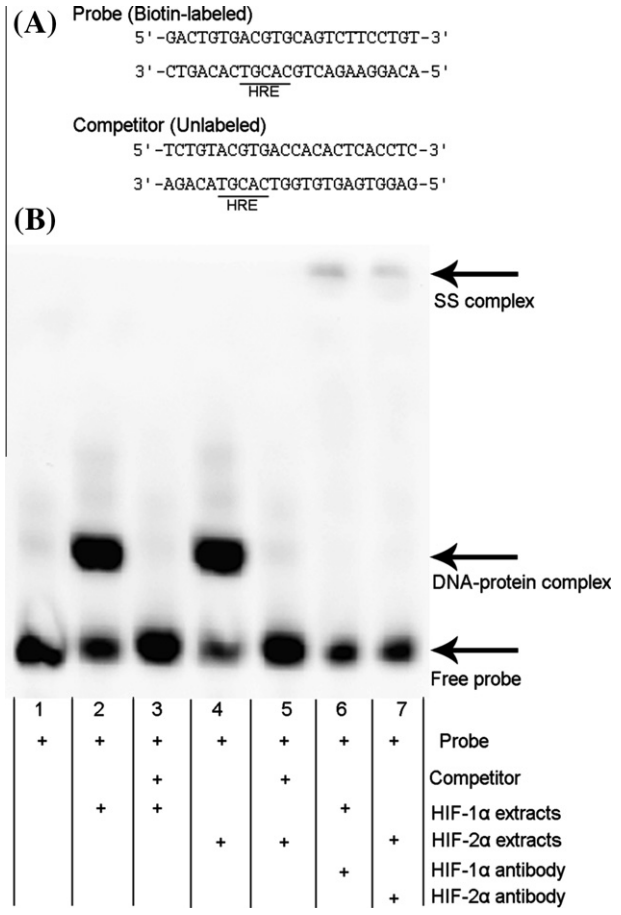



**Fig. 5.** HIF-1α and HIF-2α interacts with the HRE at the *Prox1* promoter in vitro. (A) Sequences of a biotin-labeled double strand oligonucleotide probe containing the *Prox1* HRE used for EMSA and an unlabeled oligonucleotide probe with the HIF-1α consensus binding sequence used as a competitor in EMSA. (B) EMSA. Nuclear extracts from HLE cells transfected with a HIF-1α or HIF-2α expression plasmid were incubated with the biotin-labeled EMSA probe for 20 min, and fractionated through a 5% polyacrylamide gel. The DNA–protein complex was detected by chemiluminescence. Nuclear extracts containing HIF-1α or HIF-2α resulted in formation of a DNA–protein complex (lane 2 and lane 4 in comparison to lane 1 without nuclear extracts). The HIF-1α-DNA or HIF-2α-DNA complex was eliminated by addition of 200-fold excess of the EMSA competitor (lane 3 and lane 5). The HIF-1α-DNA complex and HIF-2α-DNA complex can be shifted by a specific anti-HIF-1α antibody (lane 6) and an anti-HIF-2α antibody (lane 7). SS complex, super-shifted protein-DNA complex.

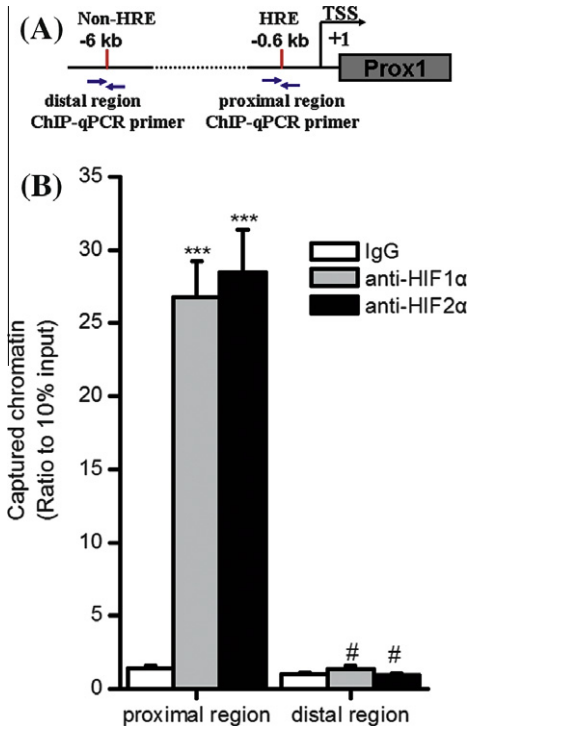



**Fig. 6.** HIF-1α and HIF-2α interacts with the HRE at the *Prox1* promoter in vivo. (A) Schematic diagram of the 5′-upstream region of the human *Prox1* gene. TSS denotes the transcription start site (+1 position). A pair of qPCR primers covering a proximal region containing the *Prox1* HRE (−640 bp to −592 bp) was used for ChIP analysis. A pair of qPCR primers covering a distal region, about 6000 bp upstream from TSS was used as a negative control for ChIP analysis. (B) ChIP analysis indicates that both HIF-1α and HIF-2α could bind specifically to the *Prox1* HRE in the proximal promoter region, but not to the distal site without an HRE. Immunoprecipitation was preformed with an anti-HIF-1α antibody or an anti-HIF-2α antibody, whereas IgG was used as control. (***$P < 0.0001$ compared to IgG; #not significant compared to IgG).

collagen matrices in mouse embryoid bodies [34]. In addition, HIF-1α was shown to correlate with expression of VEGF-C and lymphangiogenesis during wound healing, in response to inflammation, and in breast cancer [35,36]. Activation of the tyrosine kinase-linked receptor VEGFR3 has been shown to drive lymphangiogenesis induced by VEGF-C [37]. Flister et al. [33] showed that NF-kB and Prox1 can coordinately up-regulates expression of VEGFR3. Here, we identified *Prox1* as a direct target for HIF-1α and HIF-2α. We propose that hypoxia induces transcriptional activation of *Prox1* by HIF-1α and HIF-2α, which up-regulates the expression of *VEGFR3*, facilitating lymphangiogenesis. Our study may, therefore, provide on potential mechanism by which hypoxia promotes lymphangiogenesis.

*Prox1* expression has been shown to be increased in several primary cancers, including colon cancer, brain tumors (astrocytic gliomas) and Kaposi sarcoma [1]. Because the tumor microenvironment is highly hypoxic, up-regulation of *Prox1* in cancers noted above may be through the hypoxia-response mechanism identified in this study for *Prox1*. In addition, *Prox1* has been shown to be involved in the development of the central nervous system, lens, liver, pancreas, and heart [1,30]. Moreover, one major pathway for cancer metastasis is through lymphatic vessels [38]. It should be interesting to investigate the potential role of hypoxia-induced *Prox1* expression in these physiological and pathological processes in the future.

## Conflict of interest

For Q.W., he acted as a consultant for Merck; for other authors: some other research in Wang Laboratory, but not the present study, was supported partly by Merck.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.01.053.

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